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(71) [Applicant]
[Identification Number] 000010087
[Name] TOTO, LTD.
[Address] 2-1-1, Nakajima, Kokura-kita-ku, Kitakyushu-shi, Fukuoka-ken
(71) [Applicant]
[Identification Number] 390014889
[Name] Yamato Chemicals, Inc.
[Address] 5-7-12, Uehommachi, Tennoji-ku, Osaka-shi, Osaka
(72) [Inventor(s)]
[Name] Moriyama Yasushi
[Address] 2-1-1, Nakajima, Kokura-kita-ku, Kitakyushu-shi, Fukuoka-ken Inside of TOTO, LTD.
(72) [Inventor(s)]
[Name] Shimizu Yasuhiro
[Address] 5-7-12, Uehommachi, Tennoji-ku, Osaka-shi, Osaka Inside of Yamato Chemicals, Inc.
(72) [Inventor(s)]
[Name] Mitsuo Shinji
[Address] 2-3-1, Matsukadai, Higashi-ku, Fukuoka-shi, Fukuoka-ken Inside of Kyushu Sangyo University
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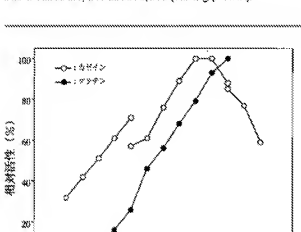
Epitome

(57) [Abstract]
[Objects of the Invention] The new alkaline protease which has powerful activity also to insoluble protein, such as not only fusibility protein but a keratin, is offered.
[Elements of the Invention] Alkaline protease which has the following physicochemical property.
(a) An operation and substrate specificity : act on protein and a peptide, and the mechanism of an end mold cuts the peptide linkage, and generate the oligopeptide and the amino acid of low molecular weight. Moreover, powerful activity is shown also to insoluble protein, such as a keratin.
(b) In stable pH:30 degree C and the processing conditions of 24 hours, it is stable at pH 1 5-12 0.
(c) Optimum pH is 11.0-11.5, when casein is made into a substrate, and when a keratin is made into a substrate, it is 12.0 or more.
(d) Specific activity : when casein is made into a substrate, it is about 1,100 (PU/mg protein), and when a keratin is made into a substrate, it is about 3,300 (KU/mg protein).



CLAIMS

[Claim(s)]
[Claim 1] Alkaline protease which has the following physicochemical property.
(a) An operation and substrate specificity : act on protein and a peptide, and the mechanism of an end mold cuts the peptide linkage, and generate the oligopeptide and the amino acid of low molecular weight. Moreover, powerful activity is shown also to insoluble protein, such as a keratin.
(b) In stable pH:30 degree C and the processing conditions of 24 hours, it is stable at pH 1.5-12 0.
(c) Molecular weight : in an SDS electrophoresis method, the average molecular weight from about 20,000 amino acid sequence is 19,150.
(d) Isoelectric point : it is 10.0 (isoelectnc focusing) or more.
(e) Specific activity : when casein is made into a substrate, it is about 1,100 (PU/mg protein), and when a keratin is made into a substrate, it is about 3,300 (KU/mg protein).
[Claim 2] Furthermore, alkaline protease according to claim 1 which has the following physicochemical property
(f) Optimum pH : optimum pH is 11.0-11.5, when casein is made into a substrate, and when a keratin is made into a

substrate, it is 12.0 or more.
(g) Optimum temperature : the optimal operative temperature is 70-75 degrees C, when casein is made into a substrate, and when a keratin is made into a substrate, it is 65-70 degrees C.
(h) Stable temperature : in the processing conditions for [pH] 7.0 or 10 minutes, it is stable to 60 degrees C irrespective of addition of calcium, and additive-free.
(i) Inhibition: Although activity is not checked by EDTA (ethylenediaminetetraacetic acid), it is prevented in PMSF (phenylmethane sulfonyl full ORAIDO) and SSI (Streptomyces subtilisin inhibitor).
[Claim 3] The gene which shows alkaline protease according to claim 1 to the array table which carries out a code, and the array number 1
[Claim 4] Alkaline protease according to claim 1 which has the amino acid sequence shown in an array table and the array number 2.
[Claim 5] Alkaline protease according to claim 1 obtained from alkalophilic Actinomyces NOKARUDIOPUSHISU (Nocardiopsis) ****
[Claim 6] NOKARUDIOPUSHISU Alkaline protease according to claim 1 obtained from ESUPI (Nocardiopsis sp.) TOA-1 share.
[Claim 7] The manufacturing method of the alkaline protease which comes to contain the process which cultivates the microorganism which belongs to an alkalophilic NOKARUDIOPUSHISU group and has alkaline protease production ability according to claim 1, and the process which separates this alkaline protease from the culture obtained at this process.
[Claim 8] The alkaline protease manufacturing method according to claim 7 to which culture of a microorganism is carried out with the alkalinity of pH 8.0-11.0 at the culture temperature of 15-35 degrees C in the first half.
[Claim 9] Said microorganism is NOKARUDIOPUSHISU. ESUPI Alkaline protease manufacturing method according to claim 7 which is TOA-1 share
[Claim 10] NOKARUDIOPUSHISU ESUPI TOA-1 share (FERM P-18676).

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] this invention -- a keratin -- it is related with the new alkalophilic Actinomyces which has new alkaline protease with high resolution, its manufacturing method, and this alkaline protease production ability.
[0002]
[Description of the Prior Art] Alkaline protease is an enzyme which hydrolyzes proteinic peptide linkage specifically in an alkali field, and is widely used in industry, such as food, fiber, leather, and a detergent. a group which it is known that such alkaline protease will be widely produced by microorganisms, such as mold, yeast, and bacteria, and is called the so-called alkalophilic microorganism -- it is produced also by the microorganism
[0003] As alkaline protease produced from the alkalophilic microorganism mentioned above, many enzymes (for example, JP,7-63366,B, JP,7-63367,B, JP,7-63368,B, etc.) obtained from the so-called alkalophilic Bacillus bacillus are already known, and development is mainly furthered as an object for detergents. Moreover, the AH-101 share alkaline protease (JP,2-255087,A) to produce, the B18-1 share alkaline protease (JP,7-63368,B) to produce are known as a thermostable enzyme obtained from the same alkalophilic Bacillus bacillus. However, it is seldom known about the protease which the alkalophilic Actinomyces which is the same alkalophilic microorganism produces, but is the enzyme (Agr.Biol Chem , 38, (1)) of the Streptomyces ongin slightly. 37-44, 1974, the alkaline protease (Biosci.Biotech.Biochem., 56, (2), 246-250, 1992) of 682 shares of Thermoactinomyces groups HS to produce, the alkaline protease (J.Appl.Bacteriol , 69, 520-529, 1990) which NOKARUDIOPUSHISUDASSOMBIREI OPC-210 produce are reported.
[0004] On the other hand, when applying a protease to detergents, it is pointed out that the enzyme which acts good also to insoluble protein, such as a keratin, is desirable (Minagawa radical: ****, 26, 322, 1985). Moreover, the case where a protease is applied to the cleaning agent of piping of an organ bath, a bath boiler, a bath floor-drain slot, and a circulation organ bath, a toilet bowl, or a washing-its-face dressing table drain requires the capacity which disassembles powerfully the insoluble protein which holds activity sufficient with the moderate temperature around 30 degrees C, and makes keratins, such as hair, dirt, or dirt, representation. In addition, since a present detergent and a present cleaning agent have the pH in an alkali field from the relation of a combination presentation, the enzyme blended with these has the optimal alkaline protease.
[0005] Furthermore, hair, feathers, etc. which use a keratin as main protein are important as a manufacture raw material of the cysteine which is the amino acid in which chemosynthesis is impossible. However, in the present conditon, the cysteine of the remarkable amount for an excessive reaction condition will be decomposed, and since yield was very bad, the mild hydrolysis approaches, such as protease processing, were desired. Furthermore, if an angle, feathers, etc. of a cow and a water buffalo which are discarded in large quantities as an unused resource in developing countries consist of useful amino acid and peptide liquid and hydrolyzed vegetable protein can be manufactured by mild hydrolysis, they are very useful for these countries as drugs or supplements, such as an infusion solution. The enzyme as a hydrolysis agent from the property for a keratin to swell in a high alkali field in these cases, and to become easy to receive an operation of an enzyme has the optimal alkaline protease.
[0006] Besides these applications, quasi drugs, such as refinement (improvement in aesthetic property) of depilation of

detergents, such as a kitchen detergent, and a detergent for tableware scrubbers, a housing detergent, and a fur (raw leather), silk, wool, leather, etc., etc., a depilation cream, and a bathing agent, a contact lens cleaning agent, and the alkaline protease which was excellent in the application of food processing, such as a texture softener, a physic, a reagent, etc., etc. at the resolving power of insoluble protein are very more useful still.
[0007] To such an application, especially, in the resolving power of insoluble protein, such as a keratin, conventional alkaline protease is inadequate, and development of the new alkaline protease which has still more powerful keratin resolving power was desired.
[0008] From such a viewpoint, this invention persons already discovered new alkaline protease, and have applied for the patent (JP,2000-060547,A). This alkaline protease has small molecular weight, and it has the outstanding proteolysis activity. However, there was a trouble that dispersion and an enzyme became [the productivity of this enzyme] large the whole culture rapidly unstable by 10.0 or more pH. Therefore, development of stable alkaline protease is desired also in the high alkali field stably possible [production of an enzyme].
[0009]
[Problem(s) to be Solved by the Invention] the keratin excellent in this invention -- it aims at offering the new alkaline protease which has resolution and the stability in a high alkali field. Moreover, this invention aims at offering the manufacturing method by which the new microorganism which produces the above-mentioned alkaline protease, and the above-mentioned alkaline protease using the microorganism were stabilized.
[0010]
[Means for Solving the Problem] the above situations -- setting -- a keratin with powerful artificers -- as a result of mainly searching an alkalophilic Actinomyces for the microorganism which produces the alkaline protease which has resolution as a core, one share of Actinomyces belonging to the NOKARUDIOPUSHISU group of good alkalinity finds out producing target new alkaline protease efficiently and stably by aerobic culture, and came to complete this invention.
[0011] That is, this invention offers the new alkaline protease which has the following physicochemical property.
[0012] (a) An operation and substrate specificity : act on protein and a peptide, and the mechanism of an end mold cuts the peptide linkage, and generate the oligopeptide and the amino acid of low molecular weight. Moreover, powerful activity is shown also to insoluble protein, such as a keratin.
(b) In stable pH 30 degree C and the processing conditions of 24 hours, it is stable by pH 1.5-12.0
(c) Molecular weight : in an SDS electrophoresis method, the average molecular weight from about 20,000 amino acid sequence is 19,150.
(d) Isoelectric point : it is 10.0 (isoelectric focusing) or more.
(e) Specific activity : when casein is made into a substrate, it is about 1,100 (PU/mg protein), and when a keratin is made into a substrate, it is about 3,300 (KU/mg protein).
[0013] moreover, the thing which comes to contain the process which cultivates the microorganism which the manufacturing method of the above-mentioned new alkaline protease by this invention belongs to an alkalophilic NOKARUDIOPUSHISU group, and has the above-mentioned alkaline protease production ability, and the process which separates this new alkaline protease from the culture obtained at this process -- it comes out. The new strain which has the above-mentioned new alkaline protease production ability by this invention further again is NOKARUDIOPUSHISU. ESUPI It is TOA-1 share (FERM P-16676).
[0014]
[Embodiment of the Invention] The alkaline protease offered by this invention is an enzyme which has the following physicochemical property in addition to the property of above-mentioned (a) - (e).
[0015] (f) Optimum pH : optimum pH is 11.0-11.5, when casein is made into a substrate, and when a keratin is made into a substrate, it is 12.0 or more.
(g) Optimum temperature : the optimal operative temperature is 70-75 degrees C, when casein is made into a substrate, and when a keratin is made into a substrate, it is 65-70 degrees C.
(h) Stable temperature : in the processing conditions for [pH] 7.0 or 10 minutes, it is stable to 60 degrees C irrespective of addition of calcium, and additive-free.
(i) Inhibition: Although activity is not checked by EDTA (ethylenediaminetetraacetic acid), it is prevented in PMSF (phenylmethane sulfonyl fluoride) and SSI (Streptomyces subtilisin inhibitor).
[0016] The alkaline protease by this invention also hydrolyzes powerfully insoluble protein, such as a keratin which was hard to be decomposed, not only in fusibility protein, such as casein, but in the conventional protease. Therefore, it is very effective if it is blended in order to raise a cleaning effect to the detergent for garments, or a softening agent and various detergents, or added by lock out removers, such as piping of an organ bath, a bath boiler, a bathroom gutter, and a circulation organ bath, a toilet bowl, and a washing-its-face dressing table drain. Furthermore, a keratin is applicable also to amino acid manufacture of the peptide from the hair used as main protein, feathers, ****, etc., a cysteine, etc. Moreover, it becomes possible to perform refinement (improvement in aesthetic property) of depilation of a fur (raw leather), silk, wool, leather, etc., etc. on milder conditions. Furthermore, it may be used in the very wide range industrial fields, such as coal chemical products, such as a depilation cream and a bathing agent, a meat softener or physic, and a reagent. Moreover, since the strain by this invention secretes the above-mentioned alkaline protease out of a fungus body efficiently, it is advantageous at the point that the production can be performed efficient at a simple process.
[0017]
[Detailed Description of the Invention] The new alkaline protease by this invention is producible using a microorganism. Especially alkaline protease according to this invention preferably is an alkalophilic NOKARUDIOPUSHISU group, especially NOKARUDIOPUSHISU. It is produced by ESUPI (Nocardiopsis sp.) TOA-1 share. This strain is separated

from the common house of Chigasaki, Kanagawa by artificers. This strain is an alkalophilic Actinomyces and has the mycology-property shown below. In addition, it was an alkalophilic microorganism, and by the usual neutral culture medium, it did not grow, or since growth was very poor, the bacteria stock used the alkaline culture medium of sodium-carbonate addition 1.0% on the occasion of examination of the following mycology-property.
[0018] the method of branching a morphology property 1 sporulation hypha, and gestalt: -- method of forming simple branching and direct-like 2 spore : The gestalt of three spore in which aerial mycelium divides and carries out a chain, and magnitude : a ***** type -- smoothing and 0.5 mumx1.0 Existence of mum grade 4 flagellum : Existence which nothing 5 spore obtains : Nothing.[0019] Physiological property 1 growth temperature requirement /pH : Liquefaction of 15 - 40 ** / 7.5 to pH 132 gelatin : it carries out (it will liquefy in three days).
3) hydrolysis of starch : the coagulation of 4 cleaning cow's milk to hydrolyze, and : which is not peptonizated : solidified -- generation of the 5 melanin Mr. coloring matter to be peptonizated in four days : Utilization nature (+ and;- which carries out utilization -- utilization is not carried out) of 6 each carbon source which is not generated
a) L-arabinose : +b D-xylose : A +cD-glucose : +dD-fructose : +e sucrose : +f inositol : +g L-rhamnose : +h raffinose : +i D-mannitol : Growth situation in +7 each culture medium (the color on a growth situation and the front face of a cluster, the color on the rear face of a cluster, diffusion coloring matter)
a) A sucrose nitrate agar medium : A fitness, white, colorlessness, thin flesh-color - pink b glucose asparagine agar medium : Fitness, white, colorlessness, a nothing c glycerol asparagine agar medium : Fitness, white, colorlessness, nothing d starch and a mineral salt agar medium : A fitness, white, colorlessness, and light-brown-color e thyrosin agar medium : Fitness, white, colorlessness, light brown color, melanin-less f nutrient agar medium : Fitness, white, colorlessness,-less g yeast and a malt-agar culture medium: Fitness, white, colorlessness,-less h oatmeal agar medium Fitness, white, colorlessness, thin flesh color - pink [0020] Bacteria stock TOA-1 share has the description of an Actinomyces on an above-mentioned mycology-property, especially an above-mentioned morphology target. Then, the group was searched according to "a classification and identification" (the edited by Society for Actinomycetes Japan, 2001) of an Actinomyces. First, since a bacteria stock contains only meso-diaminopimelic acid in a cell wall, a bacteria stock is considered to be a strain belonging to groups other than a streptomyces and KINEOSUPORIA. Moreover, although the bacteria stock had little fragmentation of radical viable cell yarn, and zigzag-like hypha, it is full of fragmentation of aerial mycelium, and it was considered to be the Actinomyces of nocardio-form.
[0021] Then, 16SrDNA(s) of a bacteria stock were prepared according to the compendium (same as the above), and homology with Actinomyces each group was searched. Consequently, the kind whose bacteria stock corresponds completely although each strain of a NOKARUDIOPUSHISU group and 94.7 - 97.6% of homology were accepted was not accepted. It is NOKARUDIOPUSHISU which has the highest homology. It was Alba. Moreover, NOKARUDIOPUSHISU which is good alkalinity like a bacteria stock 96.7% and the homology of DASSOMBIREI in the inside of a NOKARUDIOPUSHISU group were not so high.
[0022] Namely, bacteria stock TOA-1 takes that it is good alkalinity etc. into consideration, and it is NOKARUDIOPUSHISU. It is judged as one strain of a close relationship to Aiba, and is NOKARUDIOPUSHISU. ESUPI It was named TOA-1 (Nocardiopsis sp.TOA-1). In addition, a bacteria stock is the independent administrative agency National Institute of Advanced Industrial Science and Technology. It ***'s in the patent living thing deposition pin center,large also as that of trust number FERM P-18676.
[0023] Since the culture condition above-mentioned strain is an alkalophilic Actinomyces, it is necessary to perform the culture in an alkali field. As an approach for making a culture medium into alkalinity, it comes out exceptionally, and a certain need is not and should just add a sodium carbonate or a sodium hydrogencarbonate in the usual culture medium. As a carbon source, monosaccharides, such as a glucose, soluble starch, and a cellulose, a polysaccharide, etc. can be used. As a nitrogen source, inorganic substances, such as a nitrate and ammonium salt, are begun and a urea, a peptone, dry yeast, a yeast extract, skim milk, soybean powder, corn steep liquor, casein, a meat extract, amino acid, etc. are used. Various mineral salt, for example, magnesium salt, potassium salt, sodium salt, phosphate, etc. may be added if needed other than these carbon sources and nitrogen sources. As a source of alkali added to a culture medium, carbonates, such as about 0.5 - 2.0% of sodium carbonate and a sodium hydrogencarbonate, or a sodium hydroxide, ammonia, etc. can be used, and, as for pH of a culture medium, 8.0 to about 11.0 are desirable. Culture is preferably performed the culture temperature of 20-40 degrees C in such a culture medium. ***(ing) or shaking aerobically during the 2nd - the 7th at 30-35 degrees C. The new alkaline protease by this invention is the basis of the above culture conditions, and is secreted and accumulated mainly into culture medium.
[0024] independent in a known purification method, in order to extract and refine the enzyme by this invention from the extraction above-mentioned culture medium of an enzyme -- or it can use together and use. Since this enzyme is secreted mainly out of a fungus body (inside of culture medium), it can obtain crude enzyme liquid easily by removing a fungus body by filtration or centrifugal separation. setting; by organic solvents, such as a salting-out, methanol by the purification method of further known [crude enzyme / this], for example, an ammonium sulfate etc., ethanol, and an acetone, -- independent in the adsorption process; ultrafiltration; gel-filtration-chromatography; ion-exchange-chromatography; canal chromatography by a keratin etc., and other various chromatographies -- or it can use together and refine.
[0025] It will be as follows if a desirable purification method is shown. First, saturated ammonium sulfate is added 80% to culture filtrate, a salting-out is performed, and the obtained precipitate is dissolved in the buffer solution. Subsequently, a purification enzyme uniform in SDS electrophoresis can be obtained by performing ion exchange chromatography by CM-Toyopearl 650M {TOSOH CORP. make} and DEAE-Toyopearl 650M {company make}.
[0026] The property of the alkaline protease by property this invention of an enzyme is as being shown below. In addition,

an activity measurement method shall say the following approach to below.
[0027] After mixing 50mM glycine / NaCl/NaOH buffer-solution (pH11.0) 1.9 ml containing protease activity measurement method casein 0.6% with 0 1ml enzyme liquid, making it react for 10 minutes at 30 degrees C, adding a 2ml 0.11M trichloroacetic-acid solution and putting for 30 minutes at 30 degrees C, it filters by filter paper Nby ADVANTEC Co., Ltd 0.5C. Subsequently, 0.5ml of this filtrate is added to a 2.5ml 0.5M sodium-carbonate solution, 0.5ml of phenol reagents diluted further 3 times is added, after ****, it is further left for 30 minutes at a room temperature, and the absorbance of 660 nm is measured. The amount of enzymes which makes the absorbance equivalent to the tyrosin of 1microg increase in 1 minute is defined as protease activity 1 unit (1PU) by the bottom of the above-mentioned Measuring condition
[0028] 2.9ml 50mM glycine / NaCl/NaOH buffer solution (pH12.0) are added to the keratin powder (Tokyo formation) of 60mg of keratin decomposition activity measuring methods, and 0.1 moremt enzyme liquid is added, and a shaking (120rpm) is carried out for 1 hour, and it is made to react at 30 degrees C. Subsequently, 2.5ml of 0.11M trichloroacetic acids is added, a reaction is stopped, and it filters at 30 degrees C by filter paper NO[by after / 30 minute standing / ADVANTEC Co., Ltd.] 5C. 2.5ml of 0.5M sodium-carbonate solutions is added to 0.5ml of this filtrate, the phenol reagent diluted further 3 times is further left for 30 minutes at a room temperature after 0.5ml addition churning, and the absorbance of 660nm is measured. The amount of enzymes which makes the absorbance equivalent to the tyrosin of 1microg increase in 10 minutes is defined as keratin decomposition activity 1 unit (1KU) by the bottom of the above-mentioned Measuring condition.
[0029] (1) Act on an operation, substrate specificity protein, and a peptide, and the mechanism of an end mold cuts the peptide linkage, and generate the oligopeptide and the amino acid of low molecular weight. Moreover, powerful activity is shown also to insoluble protein, such as a keratin.
[0030] (2) Based on optimum pH and the activity measurement method of Stability pH above, the effect of pH exerted on this enzyme was investigated. In addition, HCl/KCl (pH 1.0-1.5), a glycine/NaCl/HCl (pH 2.0-3.0), an acetic acid (pH 4.0-5.0), a phosphoric acid (pH 6.0-7.0), a tris hydrochloric acid (pH 7.0-9.0), a glycine / NaCl/NaOH (pH 9.0-12.0), and KCl/NaOH (pH 12.0-13.0) were used as the buffer solution. The relative activity in each pH at the time of setting maximum of activity to 100 was shown in Fig. 1. Fig. 1 shows that the optimum pH of this enzyme is 11.0-11.5 when casein is made into a substrate in 30 degrees C, and it is 12.0 or more when a keratin is made into a substrate. The pH stability of this enzyme was similarly shown in Fig. 2. After holding this enzyme at 30 degrees C in the buffer solution of each pH for 24 hours, the residual protease activity was shown as relative activity which set unsettled enzyme activity to 100. Fig. 2 shows that this enzyme is stable in very wide range pH region to pH 1.5-12.0 under the above-mentioned processing condition.
[0031] (3) According to optimum temperature and the stable temperature above-mentioned activity measurement method, the effect of the temperature exerted on this enzyme was investigated. The relative activity in each temperature at the time of setting the maximum activity to 100 was shown in Fig. 3. The optimum temperature of this enzyme is 70-75 degrees C, when casein is made into a substrate, and Fig. 3 shows that it is 65-70 degrees C, when a keratin is made into a substrate. Moreover, after adding this enzyme to the 100 mM tris hydrochloric-acid buffer solution (pH7.0) and holding for 10 minutes under the 40-80-degree C monograph affair of the range, the residual protease activity was measured. The result was shown in Fig. 4. Fig. 4 shows that this enzyme is stable to 60 degrees C. In addition, the effectiveness of calcium addition (10mM) was not accepted about the temperature stability of this enzyme
[0032] (4) Molecular weight was about 20,000 when the molecular weight of a molecular weight book enzyme was measured with the SDS electrophoresis method. Moreover, the array table mentioned later and the average molecular weight computed from the amino acid sequence of array number 2 publication were 19,150.
[0033] (5) When the isoelectric point of an isoelectric point book enzyme was measured with isoelectric focusing, the isoelectric point was 10.0 or more.
[0034] (6) The specific activity of a specific activity book enzyme was measured according to the activity measurement method. In addition, protein concentration hydrolyzed the enzyme with the hydrochloric acid, and computed it by carrying out the quantum of the generated amino acid by the ninhydrin method. The enzyme used the purification preparation uniform in electrophoresis. Consequently, the specific activity of this enzyme was about 1,100 (PU/mg protein), when casein was made into a substrate, and when a keratin was made into a substrate, it was about 3,300 (KU/mg protein).
[0035] (7) inhibition -- PMSF (phenylmethane sulfonyl full ORAIDO) which is common enzyme inhibitor EDTA (ethylenediaminetetraacetic acid) And about SSI (Streptomyces subtilisin inhibitor), these investigated the effect affect the activity of this enzyme. Each inhibitor was dissolved in 50mM tris hydrochloric-acid buffer solution (pH9.0) so that it might become predetermined concentration, and processing was performed for 30 minutes at 30 degrees C after adding this enzyme. Subsequently, the constant rate was isolated preparatively from the processing solution and the residual activity was measured according to the activity measurement method. Consequently, this enzyme was checked by PMSF and SSI and did not receive inhibition by EDTA. From this, it became clear that this alkaline protease was a serine protease
[0036] (8) The amino terminal sequence of an amino terminal sequence book enzyme was determined using the gaseous-phase amino acid sequence analyzer (the Shimadzu make, PPSQ-21). The array from the amino terminus of this enzyme to the 25th was shown below.
Ala-Asp-Ile-Ile-Gly-Gly-Leu-Ala-Tyr-Thr-Met-Gly-Gly-Arg-Cys-Ser-Val-Gly-Phe-Ala-Ala-Thr-Asn-Ala-Ser [0037] (9) According to protocols, such as a base sequence, amino acid sequence compendiums (for example, J.Sambrook, E.F.Fritsch, T.Maniatis: Molecular Cloning A Laboratory Manual, 2nd ed Cold Spring Harbor Laboratory Press, 1989, etc) and a used device, and a reagent kit, the gene of this enzyme and the array of amino acid were determined. First,

the local array of this enzyme was determined in order to design a primer. The purification enzyme was disassembled by the lysyl proteinase after urea processing (Wako Pure Chem), and the amino acid sequence was determined for the obtained fragmentation by the gaseous-phase amino acid sequence analyzer. From the suitable local array and suitable amino terminal sequence which were acquired here, two kinds of oligonucleotide primers were compounded with the phospho aminodite method. The gene was amplified by this primer by PCR (the product made from Biometra, and T-Gradient Thermoblock 050-801). Consequently, the specific magnification fragment was accepted before and after 0.5kbp. The gene of the perfect length who does the code of this enzyme from the genomic library of bacteria stock TOA-1 by using this fragment as a probe was screened. The DNA sequencer (the product made from LICOR, LICOR-4000) which makes a principle a dideoxy chain termination method (5467 F. Sanger et al. and Proc. Natl. Acad. Sci., 74, 5463-1977) determined the base sequence of the obtained clone. The base sequence (564bp) was shown in the array table and the array number 1. Moreover, the amino acid sequence (188 amino acid) was determined based on the base sequence, and the amino acid sequence was shown in the array table and the array number 2.
[0038]
[Example] Next, although the following examples explain this invention to a detail further, this invention is not limited to these.
[0039] Example 1 Preparation NOKARUDIOPUSHISU of crude enzyme powder ESUPI In 50ml (30 degrees C, shaking culture during three days) of TOA-1 share preculture liquid, they are skim milk 0.5% and a yeast extract. 0.1% and sodium carbonate which sterilized another and was added Inoculation was carried out to the small jar fermenter into which 4000ml (pH10.5) of culture media containing 1.0% was put, and it cultivated by quantity-of-airflow 1 v/v/min and rotational frequency 200rpm for three days at 30 degrees C. After culture termination, at-long-intervals alignment separation of the culture medium was carried out by 8000 rpm for 10 minutes, and the fungus body was removed. This obtained 3,800ml of 30 PU/ml crude enzyme ****.
[0040] Example 2 To the small jar fermenter into which the 4000ml of the same culture media as the preparation example 1 of a purification enzyme was put, it is NOKARUDIOPUSHISU. ESUPI Inoculation of the 50ml of the TOA-1 share preculture liquid was carried out. After cultivating this like an example 1, 3,790ml of culture supernatants was obtained according to centrifugal separation. The protease activity in pH11.0 of this digestive liquor was 29 PU(s)/ml. Subsequently, in addition, after [standing] 8000rpm performed centrifugal separation to the dark place at 5 degrees C one whole day and night, and precipitate was collected until it became saturation to this digestive liquor 80% about ammonium-sulfate powder. This precipitate was dissolved in 10mM phosphate buffer solution (pH7.0), and it dialyzed to this buffer solution using the cellulose tube. An enzyme is made to adsorb after dialysis through CM-Toyopearl 650M column which equilibrated the liquid in the dialysis with 10mMMOPS buffer solution (pH7.5), and it is 0-0.5M. Elution was carried out by the NaCl concentration gradient. The activity fraction was passed after dialyzing an activity fraction through DEAE-Toyopearl 650M column which equilibrated with 10mM tris hydrochloric-acid buffer solution (pH9.0), and impure protein was made to adsorb. By purification of a top Norikazu ream, 17ml (specific activity: 1,100 PU/mg) of 1940 PU/ml purification alkaline protease was obtained, and the recovery of enzyme activity was 30%. This fraction showed the single band in SDS electrophoresis, and it was checked that it is uniform as enzyme protein.
[0041] Example 3 Culture by the shaking culture and the small jar fermenter using a flask was performed using the same culture medium as the productivity example 1 of an enzyme, and the productivity of the enzyme for every culture batch was compared. First, 100ml of culture media was put into 500ml ******* flask, one platinum loop of TOA-1 share spores was inoculated from the agar medium, and shaking culture was performed for four days by 30 degrees C and 130rpm. Protease activity was measured for this culture per each culture medium in 10 ream deed and a flask. Moreover, 5 ream deed and the protease activity for every batch were independently measured for the culture using the completely same small jar fermenter as an example 1. Consequently, by flask culture, it was set to 28 to 33 PU/ml by 38 to 41 PU/ml, and small jar fermenter culture, and there was almost no dispersion in the activity by the culture batch. It became clear from this that the productivity of this enzyme was stable.
[0042]
[Layout Table]
SEQUENCE LISTING<110> TOTO Ltd.<120> Novel Alkaline-Protease-and Method-for-Preparing the Same<130> K1020658<160> 1<210> 1<211> 564<212> DNA<213> Nocardiopsis sp. TOA-1<400> 1gcc gac atc atc ggt ggc ctc gcc tac acc atg ggc gga cgc 42Ala Asp Ile Ile Gly Gly Leu Ala Tyr Thr Met Gly Gly Arg 1 5 10 tgc tcg gtc ggc ttc gcg gcc acc aac gcc tcc ggc cagccc 84 Cys Ser Val Gly Phe Ala Ala Thr Asn Ala Ser Gly Gln Pro15 20 25 ggc ttc gtc acc gcc ggc cac tgc ggc agc gtg ggg acc cag 126 GlyPhe Val Thr Ala Gly His Cys Gly Ser Val Gly Thr Gln 30 35 40 gtc agc atc ggc aacggc agg ggc gtc ttc gag cgc tcc gtc 168 Val Ser Ile Gly Asn Gly Arg Gly Val Phe Glu Arg Ser Val 45 50 55 ttc ccg ggc aac gac gcc gcc ttc gtc cgg ggc acg tccaac 210 Phe Pro Gly Asn Asp Ala Ala Phe Val Arg Gly Thr Ser Asn 60 65 70 ttc acc ctg acc aac ctg gtc agc cgc tac aac agc ggc ggc 252 Phe Thr Leu Thr Asn Leu Val Ser Arg Tyr Asn Ser Gly Gly 75 80 tac gcc acc gtc tcg ggc tcc agc acg gcg ccc atc ggt tcg 294 Tyr Ala ThrVal Ser Gly Ser Ser Thr Ala Pro Ile Gly Ser85 90 95 cag gtc tgc cgc tcc ggc tcc acc acc ggc tgg tac tgc ggc 336 Gln Val Cys Arg Ser Gly Ser Thr Thr Gly Trp Tyr CysGly 100 105 110 acc att cag gcc cgc aac cag acg gtgagc tac ccg cag ggc 378 Thr Ile Gln Ala Arg Asn Gln Thr Val Ser Tyr Pro Gln Gly 115 120 125 acc gtc cac agc ctg acc cgg acc tcc gtg tgc gcc gag ccc 420 Thr Val His Ser Leu Thr Arg Thr Ser Val Cys Ala Glu Pro 130 135 140 ggc gac tcc gcg ggctcg ttc atctcc gga acc cag gcc cag 462 Gly Asp Ser Ala Gly Ser Phe Ile Ser Gly Thr Gln Ala Gln 145 150 ggc gtg acc tcc ggc ggc tcc ggc aac tgc cgc acc ggt ggc 504 Gly Val Thr Ser Gly Gly Ser Gly Asn Cys Arg Thr Gly Gly155 160165 acg acc ttc tac cag gag gtc aac ccc atg ctc aac tcc tgg 546 Thr Thr Phe Tyr Gln Glu Val Asn Pro Met Leu Asn Ser Trp 170 175 180 ******, ******, ******, ******, ******, ******, ************ ****(1), ******, ****(1), ******, ******, **** machine ****** [0043]

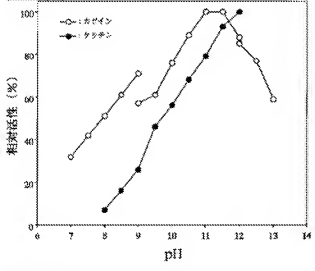
<210> 2<211> 188<212> PRT<213> Nocardiopsis sp. TOA-1<400> 2Ala-Asp-Ile-Ile-Gly Gly Leu Ala Tyr Thr-Met-Gly-Gly-Arg 1 5 10Cys Ser Val Gly Phe Ala Ala Thr Asn Ala-Ser-Gly-Gln-Pro 15 20 25Gly Phe Val Thr Ala Gly His Cys Gly Ser Val Gly ThrGln 30 35 40 Val Ser Ile Gly Asn Gly Arg Gly Val Phe Glu Arg Ser Val 45 50 55 Phe Pro Gly Asn Asp Ala Ala Phe Val Arg Gly Thr Ser Asn 60 65 70 PheThr Leu Thr Asn Leu Val Ser Arg Tyr Asn Ser Gly Gly 75 80 Tyr AlaThr Val SerGly Ser Ser Thr Ala Pro Ile Gly Ser 8590 95 Gln Val Cys Arg Ser Gly Ser Thr ThrGly Trp Tyr CysGly 100 105 110 Thr Ile Gln Ala Arg Asn Gln Thr Val Ser Tyr Pro Gln Gly 115 120 125 Thr Val His Ser Leu Thr Arg Thr Ser Val Cys Ala Glu Pro 130 135 140 Gly Asp Ser Ala Gly Ser Phe Ile Ser GlyThr Gln AlaGln 145 150 Gly Val Thr Ser Gly Gly Ser Gly AsnCys Arg Thr Gly Gly155 160 165 Thr Thr Phe Tyr Gln Glu Val Asn Pro Met Leu Asn Ser Trp170175 180 Asn Leu Arg Leu Arg Thr 185 188

DESCRIPTION OF DRAWINGS

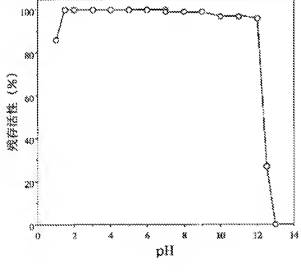
[Brief Description of the Drawings]
[Drawing 1] Drawing 1 is a graph which shows the optimum pH of the alkaline protease by this invention.
[Drawing 2] Drawing 2 is a graph which shows the stability pH of the alkaline protease by this invention.
[Drawing 3] Drawing 3 is a graph which shows the optimum temperature of the alkaline protease by this invention.
[Drawing 4] Drawing 4 is a graph which shows the stable temperature of the alkaline protease by this invention.

DRAWINGS

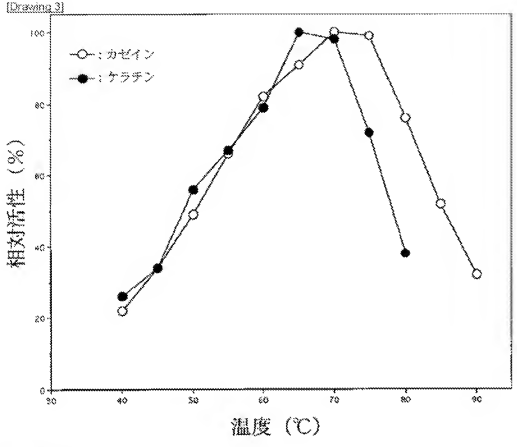
[Drawing 1]



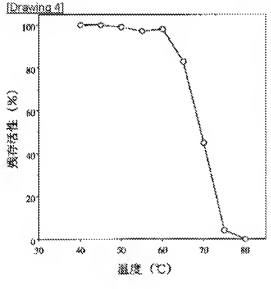
[Drawing 2]



[Drawing 3]



[Drawing 4]



[Translation done.]